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INTRODUCTION

Anti-estrogens such as tamoxifen are important therapeutic agents in the treatment and chemoprevention of breast cancers. Other compounds such as phytoestrogens, fatty acid amides such as anandamide and retinoid X receptor (RXR) agonists are also effective against breast cancer in cell lines and in animal models. Because these compounds are unrelated, it has not been appreciated that they might act through a common mechanism. These compounds all share the ability to activate a heterodimer of the steroid and xenobiotic receptor (SXR) and RXR. Our hypothesis is that SXR serves as a common molecular target for some of the anti-proliferative effects of these compounds and that activation of SXR is itself anti-proliferative. We have detected SXR protein in breast cancer cell lines, and have shown that either SXR activators or a constitutively active form of SXR (VP16-SXR) are able to slow the proliferation of breast cancer cell lines. We have found that SXR activators share the ability to induce the expression of the SXR target gene, inducible nitric oxide synthase, in breast cancer cells. This leads to an increased production of intracellular reactive nitrogen species that has been shown to be a cellular stress that can stabilize and activate the p53 tumor suppressor. In SXR activator-treated breast cancer cells, we found that expression of p53, and the expression of the p53 target genes p21 and BAX, were increased, but not until 72 hours of treatment. Apoptosis occurred in breast cancer cells treated with SXR activators beginning at 48 hours, and this is likely the reason for the observed decrease in cell proliferation. p53 activation in response to cellular stress from increased reactive nitrogen species provides a plausible mechanism to explain the apoptotic response and decreased cell proliferation in the presence of SXR activators.

BODY

Task 1. To determine whether the anti-proliferative effects of phytoestrogens, fatty amides, anti-estrogens and RXR agonists are due to activation of SXR/RXR in breast cancer cells by these compounds. (months 1-24)

a. Survey breast cancer cell lines for SXR expression.

Using a peptide antibody developed in our laboratory, Western blotting of protein lysates from various breast cancer cell lines demonstrated that SXR was expressed in the breast cancer cell lines used in this study (Fig. 1).

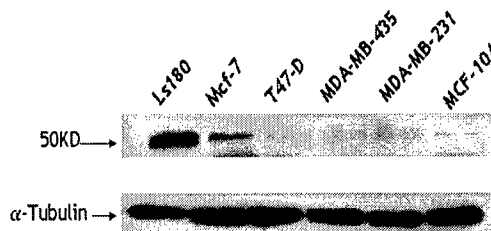


Figure 1 – Expression of SXR protein in breast cancer cell lines

Localization of SXR mRNA and protein in breast biopsies.

We examined expression of SXR in 48 breast carcinomas using immunohistochemistry and quantitative RT-PCR. We then screened possible target genes of SXR using microarray system in carcinoma cells captured by laser micro scissors. SXR was detected in carcinoma tissues but not in normal and stromal cells of breast cancer tissues. A significant positive correlation was detected between SXR labeling index and both histological grade and lymph node status of the cases. In addition, SXR mRNA level was positively correlated with CYP3A4 and MDR1 mRNA levels. Results suggest evaluation of SXR status including its target genes such as OATP-A as a predictor for prognosis and for responses to therapy of breast carcinoma patients (Y. Miki et al., manuscript submitted).

b. Transiently transfect cells with VP16-SXR and DsRED and analyze effects on proliferation after FACS separation of transfected cells.

MCF-7 cells transiently transfected with a constitutively active form of SXR, VP16-SXR, demonstrated decreased proliferation compared with control transfected cells. This result suggested that SXR transcriptional activation could slow the proliferation of breast cancer cell lines (2003 Progress Report, Figure 1).

c. Construct stable cell lines expressing VP16-SXR under the control of an inducible promoter.

Construction of VP16-SXR inducible breast cancer cell lines was attempted and was met with several technical difficulties (see 2003 Progress Report). Final analysis of strain construction led to the conclusion that basal transcription from the promoters of vectors used to make the stable lines produced non-negligible amounts of the very potent and stable transcriptional activator VP16-SXR.

d. Test whether the induction of VP16-SXR mimics the anti-proliferative activity of SXR activators.

This aim was not able to be completed due to the technical difficulties encountered in part d. above. See 2003 Progress Report for experimental details.

e. Test whether the inhibition of SXR activation with ET-743 blocks the anti-proliferative effects of SXR activators.

We applied to obtain the SXR antagonist ET-743 from its commercial manufacturer PharmaMar, and to date, have been unsuccessful in obtaining this compound. PharmaMar is not filling requests for this compound as it is being tested in clinical trials.

Task 2. Define the mechanism by which SXR activation inhibits the proliferation of breast cancer cells (months 12-36).

a. Bioinformatic analysis of public databases to detect potential SXR target genes.

This was not performed owing to our success identifying target genes using microarray analysis.

b. Microarray analysis of mRNAs from breast cancer cell lines treated with SXR activators.

Two separate microarray experiments were performed using MCF-7 and MBMDA231 breast cancer cell line RNAs. Experimental details and lists of up and down regulated genes are described in our 2004 Progress Report.

c. Chromatin immunoprecipitation and microarray analysis.

Chromatin immunoprecipitation experiments were not performed. However, extensive microarray analysis was performed using the three different datasets obtained and described in our 2004 Progress Report. Briefly, a small number of genes were found to be commonly up-regulated by treatment of either the ER⁺ line MCF-7 and ER⁻ line MBMDA231. Two of

the genes, ABC1 and INSIG1 are known SXR targets. For the remainder of the common genes, no obvious direct connection between SXR activation and a decrease in cell proliferation or increase in apoptosis could be determined.

Our analysis of the genes up or down regulated in the MCF-7 cell line led to the identification of several genes involved in apoptosis. We showed that at least one of these, TR3/NUR77, exhibited increased expression in breast cancer cell lines treated with various SXR activators (see below).

d. Screening of CpG island libraries for SXR target genes.

Although CpG island libraries were not screened during the tenure of this grant, SXR target genes were found amongst the up regulated gene lists resulting from the microarray analysis of MCF-7 and MBMDA231 cells treated with SXR activators (2004 Progress Report, Tables 7 & 9). In addition we have shown that inducible nitric oxide synthase (iNOS, NOS2A), a direct SXR target gene, plays a direct role in the mechanism by which SXR activation is able to decrease the proliferation of breast cancer cell lines and induce their apoptosis.

e. Testing of putative SXR-responsive promoters and analysis of target gene regulation.

Described in detail below.

Apoptosis assays and flow cytometry analysis

Treating the breast cancer cell lines MCF-7, T47D, MDAMB231 or MDAMB435 with SXR activators leads to a decrease in proliferation of those cells. Decreased proliferation could be due to apoptosis, necrosis or cell-cycle arrest. To sort out which of these possibilities was responsible for the observed effects, we focused on differentiating between apoptosis and necrosis. As detailed our 2004 Progress Report, we used the Cell Death Detection Elisa Plus (Roche) to detect

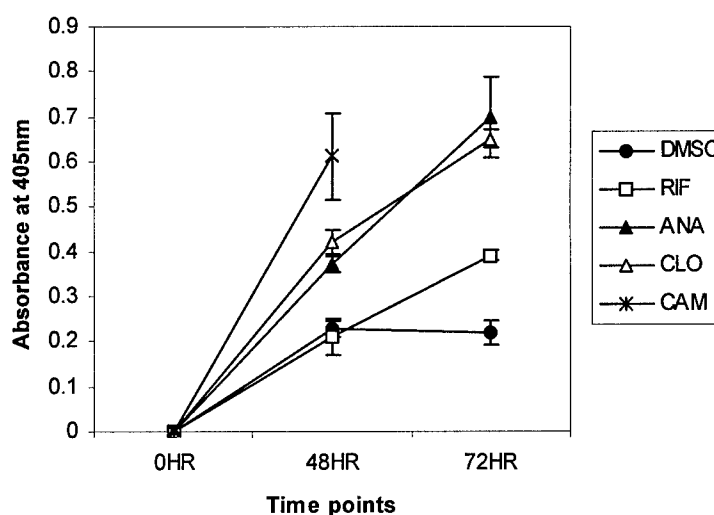


Figure 2. Apoptosis ELISA. DNA fragmentation in cell lysates from MCF-7 cells treated with 10 μ M CAMP, RIF, ANA or CLO for 48 or 72 hours. Absorbance units indicate apoptosis.

apoptosis and necrosis in a single experiment. We treated MCF-7 cells with either 10 μ M or 100 μ M of the SXR activator RIF or the apoptosis inducer camptothecin (CAM) as a positive control. We found that 10 μ M RIF was able to induce apoptosis sometime between 24 and 72 hours of treatment. In order to better determine the timing of apoptosis induction by SXR activators, we treated MCF7 cells with 10 μ M RIF, as well as the other SXR activators anadamide (ANA) and clotrimazole (CLO). Once again we used CAMP as a positive control. As shown in Figure 2, we found that RIF, anadamide and clotrimazole were all able to induce apoptosis in MCF-7 cells compared to solvent only and that the induction occurred by 48 hours of treatment.

A decrease in cell proliferation could also be caused by cell cycle arrest. It is already known that the SXR activator clotrimazole can cause cell cycle arrest in the G1 phase and inhibit proliferation of breast cancer cells (1, 10).

Therefore, we also wanted to study the effects of several SXR activators on the cell cycle.

MCF-7 cells were treated with 10 μ M RIF, anadamide or clotrimazole from 24 hours to 72 hours. DNA was stained with propidium iodide followed by flow cytometry analysis.

Mimosine was used as a positive control compound that causes G1 arrest (4). As shown in Table 1, we found that treatment with the SXR activators as a whole did not cause a consistent cell cycle arrest. We were able to detect a G1 arrest for clotrimazole and mimosine consistent with published results.

These data combined with the apoptosis studies suggest that the decrease in proliferation observed in the breast cancer cell lines treated with SXR activators was ultimately due to cell death via apoptosis.

Sample	%G1	%S	%G2
24HR			
DMSO	40.5	37.1	20.7
RIF	41.1	37.4	18.5
ANA	34.6	42	19.6
CLO	52	30.1	13.9
MIM	66.8	20.3	2.27
48HR			
DMSO	54.6	29.4	15.7
RIF	56	27.5	14.8
ANA	55.4	28.5	17.4
CLO	65.8	21	12.4
MIM	74	18.5	1.63
72HR			
DMSO	76.9	14.4	9.92
RIF	75.1	16.1	9.6
ANA	76.3	15.5	10.1
CLO	82.8	9.05	7.76
MIM	77.4	18.8	3.88

Table 1. Flow cytometry results of 24, 48 and 72 hour treatments with 10 μ M SXR activators rifampicin (RIF), anadamide (ANA) or clotrimazole (CLO). Mimosine (MIM) is used as a positive control.

Analysis of putative SXR target genes.

In order to determine the SXR-dependent mechanism responsible for apoptosis in the breast cancer cell lines, we turned to the results of microarrays performed in MCF-7 cells treated with different SXR activators (2004 Progress Report, Table 1). In the list of genes up-regulated in MCF-7 cells we found the pro-apoptotic TR3 orphan receptor which is also known as NUR77. In response to apoptotic stimuli, TR3 is translocated from the

nucleus to the cytoplasm, where it targets mitochondria to induce cytochrome c release and apoptosis (6). BCL2 interacts with TR3/NUR77, which is required for cancer cell apoptosis induced by many anti-neoplastic

agents (7). Interaction with BCL2 was required for TR3/NUR77 mitochondrial localization and apoptosis. TR3/NUR77 binding induces a conformational change in BCL2 that exposes its BH3 domain, resulting in conversion of BCL2 from a protector to a killer or from anti-apoptotic to pro-apoptotic.

These findings linked TR3/NUR77 with the BCL2 apoptotic machinery and demonstrated that BCL2 can display opposing phenotypes induced by interactions with proteins

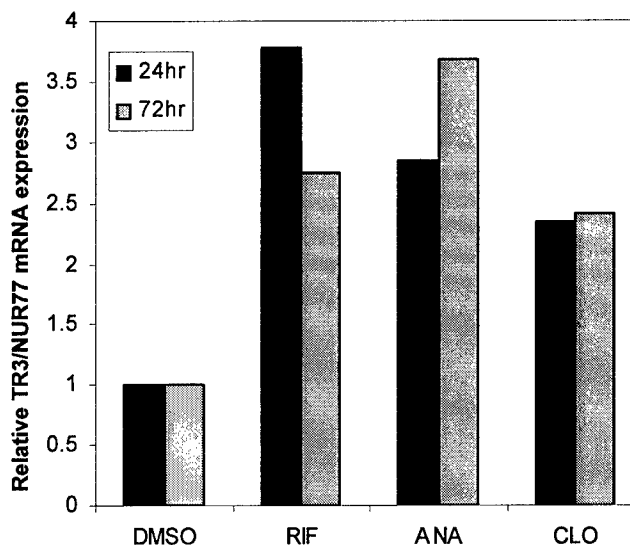


Figure 3. TR3/NUR77 expression in treated MCF-7 cells. MCF-7 cells were treated for 24 or 72 hours with 10 μ M RIF, ANA or CLO. Total RNA was harvested, reverse transcribed and analyzed by QRT-PCR.

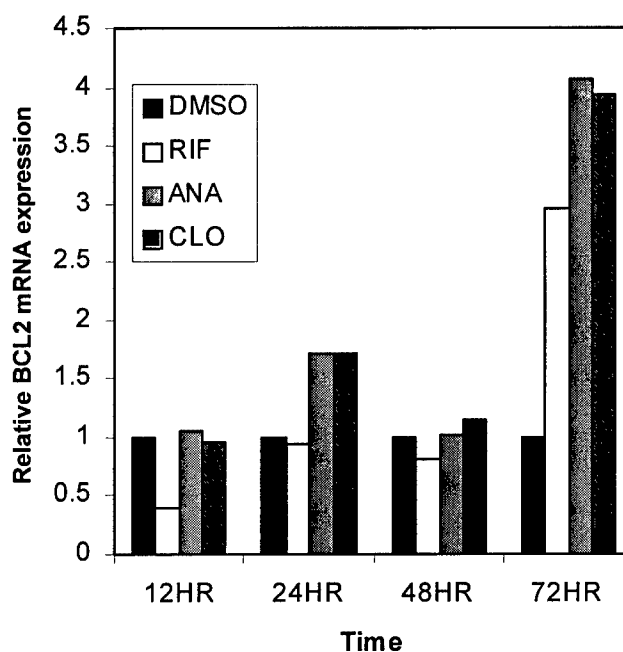


Figure 4. BCL2 expression in treated MCF-7 cells. MCF-7 cells were treated for 24, 48 or 72 hours with 10 μ M RIF, ANA or CLO. Total RNA was harvested, reverse transcribed and analyzed by QRT-PCR.

such as TR3/NUR77. To determine if effects on TR3/NUR77 and BCL2 were occurring in the breast cancer cells exposed to SXR activators, we analyzed TR3/NUR77 and BCL2 expression in MCF-7 cells treated with RIF, tamoxifen, anadamide, clotrimazole and RU-486 for 24, 48 and 72 hours using quantitative real time PCR (QRT-PCR). Briefly, RNA is isolated from treated cells using Trizol (Invitrogen). For QRT-PCR analysis, 1 μ g of total RNA was reverse transcribed using Superscript II (Invitrogen), and gene-specific primers were used to detect TR3/NUR77 or BCL2 expression which was normalized to GAPDH expression. As shown in Figure 3, we found that TR3/NUR77 expression was increased at 24 hours of treatment with any of the SXR activators. Interestingly, BCL2 expression was also increased by treatment with all of the SXR activators, but not until 72 hours of treatment (Fig. 4).

The results described above lead us to examine the expression of other proteins that play direct roles in apoptosis. BCL2 over-expression can protect cells from apoptosis. However, in our experiments, treated breast cancer cells had detectible levels of apoptosis at times before and at 72

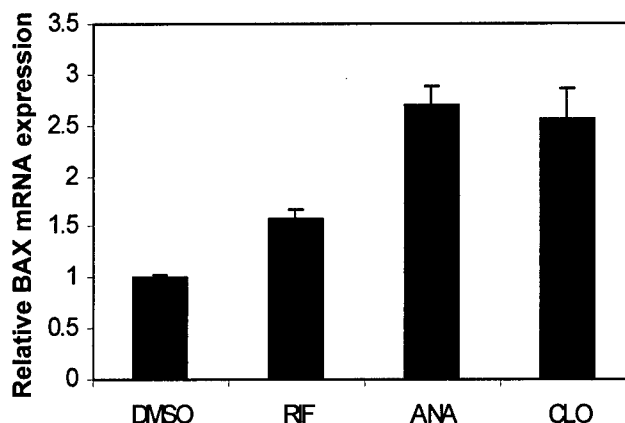


Figure 5. BAX expression in treated MCF-7 cells. MCF-7 cells were treated for 72 hours with 10 μ M RIF, ANA or CLO. Total RNA was harvested, reverse transcribed and analyzed by QRT-PCR.

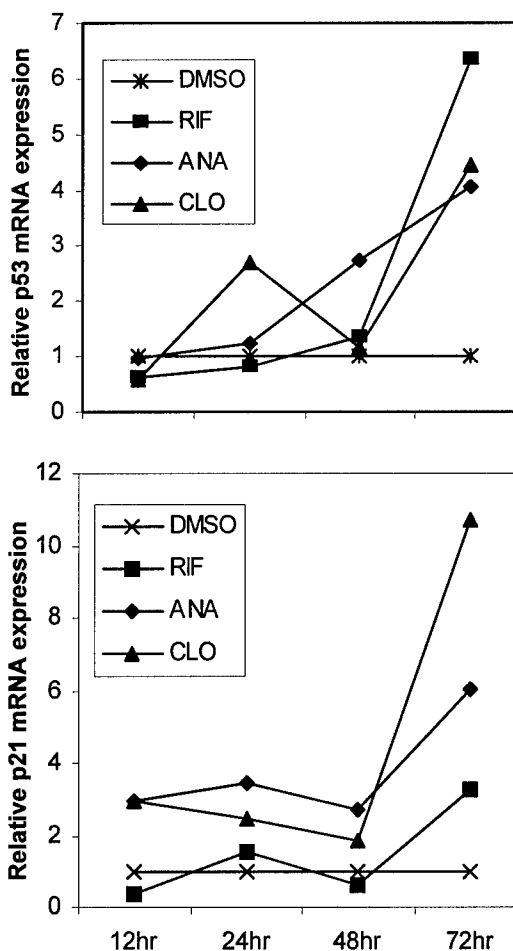


Figure 6. p53 and p21 expression in treated MCF-7 cells. MCF-7 cells were treated for 24, 48 or 72 hours with 10 μ M RIF, ANA or CLO. Total RNA was harvested, reverse transcribed and analyzed by QRT-PCR.

hours. Therefore, BCL2 levels were not serving to protect cells from apoptosis at our 72 hour treatment. The ratio of BCL2 to BAX is also key in determining cellular fate, and whether or not a cell enters apoptosis following a particular stimulus, and BCL2 associates in vivo with BAX. When BAX predominates, programmed cell death is accelerated, and the death repressor activity of BCL2 is countered (9). Therefore, we also looked at expression of BAX at the 72 hour timepoint by QRT-PCR. As shown in Figure 5, we found that BAX expression was also increased by all SXR activator treatments. Therefore, by 72 hours of treatment with SXR activators, apoptosis is occurring and could be due to BAX expression blocking the anti-apoptotic, protective effect of any BCL2 that is being expressed.

BAX is a target gene of the p53 tumor suppressor (8) and has important roles in induction of apoptosis. Cell fate decisions ultimately leading to endpoints such as apoptosis could result from changes in p53 activation as a response to a variety of cellular stresses. In addition, activated or stabilized p53 is able to increase its own expression. Therefore, we decided to examine the expression of p53 itself as well as an additional p53 target gene, p21 in breast cancer cells treated with SXR activators (2). We found that p53 expression was increased by treatment of MCF-7 cells with all SXR activators tested, but not until 72 hours of treatment (Fig. 6). Consistent with this result, we also found that expression of the p53 target gene p21 was also increased. In the case of p21, increased expression was detected as early as 24 hours after treatments, suggesting that stabilization of p53 could be the cause of the this early increase in p21 (Fig. 6).

The increase in several p53 target genes including p21, p53 and BAX seen after 72 hours of treatment with SXR activators seemed to indicate that p53 activation and the resulting changes in p53 target gene expression were a secondary response to a primary effect caused by the SXR activators. The p53 tumor suppressor can be activated in response to DNA damage or other

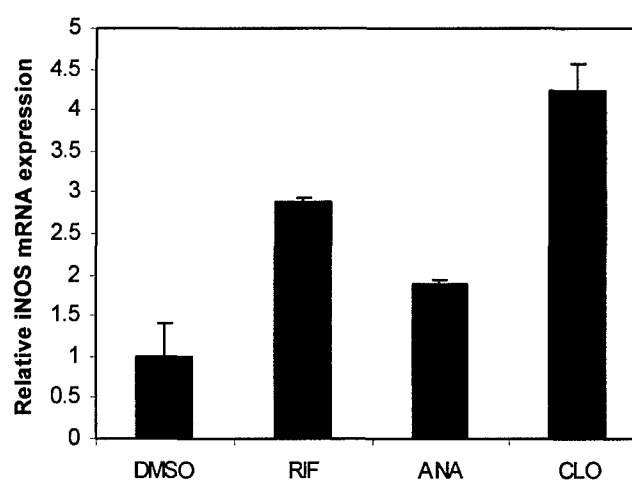


Figure 7. Inducible nitric oxide synthase (iNOS) expression in treated MCF-7 cells. MCF-7 cells were treated for 24 hours with 10 μ M RIF, ANA or CLO. Total RNA was harvested, reverse transcribed and analyzed by QRT-PCR.

cellular stress such as accumulation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) (3). In fact, RNS-stabilized p53 has been shown to up-regulate both p21 and BAX (5, 11). In our experiments, increases in p21 expression seen as early as 24 hours post-SXR activator treatment in the absence of such early increases in p53 expression are consistent with a stabilization of p53. This lead us to consider the possibility that treatment with the various SXR activators was commonly increasing a cellular stressor such as RNS. Work by Toell et al. (2002) had demonstrated the existence of SXR DNA response elements in the promoter for inducible nitric oxide synthase (iNOS, NOS2A), linking the induction of iNOS with SXR activation (12). Production of iNOS increases the cellular levels of nitric oxide (NO) and RNS. To confirm the fact that iNOS was being induced in the breast cancer cells treated with various SXR activators, we examined expression of iNOS using QRT-PCR analysis of RNA from MCF-7 cells treated with RIF, clotrimazole or anadamide for 24 hours. We found that each of the SXR activators was able to increase iNOS expression (Fig. 7). Experiments are currently underway to measure the actual intracellular increase in nitrite (NO⁻) production in MCF-7 cells following treatment with SXR activators. We anticipate that we will find an increase in NO production in the treated cells. This last experimental detail will confirm the model we propose for the mechanism by which SXR activators are able to decrease the proliferation of breast cancer cells (Fig. 8).

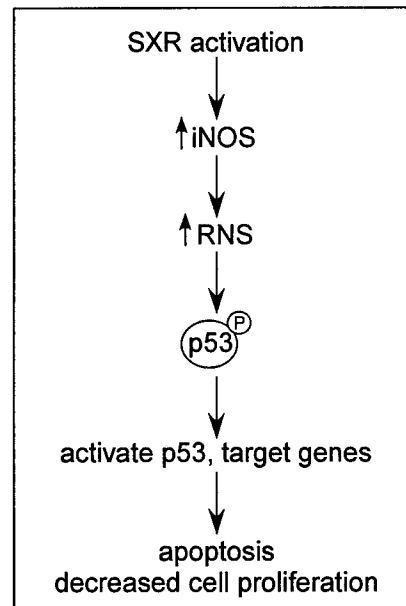


Figure 8. Proposed mechanism for SXR activation leading to apoptosis and decreased cell proliferation.

KEY RESEARCH ACCOMPLISHMENTS

- Apoptosis assays demonstrated that treatment of MCF-7 cells with various SXR activators consistently induced apoptosis starting at 48 hours. Therefore apoptosis is the likely cause of the observed decrease in breast cancer cell proliferation with SXR activator treatment.
- Further analysis of putative SXR target genes responsible for the induction of apoptosis demonstrates an increase in activated p53 as the likely cause of the apoptotic response, but p53 activation is not likely to be a direct target of SXR activation.
- Expression of the direct SXR target gene inducible nitric oxide synthase (iNOS) is increased in MCF-7 cells treated with all SXR activators. Production of nitric oxide and reactive nitrogen species has been shown to stabilize p53, and is the likely cause of the downstream apoptosis observed.
- Identification of a mechanism whereby SXR activation leads to increases in RNS, stabilization and activation of p53 and eventual cell apoptosis.

REPORTABLE OUTCOMES

Presentations:

- Dept. of Stem Cell Research, Pochon Cha Medical School, Seoul, Korea (8/2004)
- National Institute of Health Sciences, Tokyo, Japan (8/2004)
- UCI Cancer Center Retreat (11/2004)

Publications:

- Miki Y., Suzuki T., Tazawa C., Blumberg B., Sasano H. (2005) Steroid and xenobiotic receptor (SXR), cytochrome P450 3A4 and multidrug resistance gene 1 in human adult and fetal tissues. *Mol Cell Endocrinol.* 231, 75-85.
- Fujimura, T., Takahashi, T., Urano, T., Kumagai, J., Ogushi, T., Horie-Inoue, T., Ouchi, Y., Kitamura, T., Muramatsu, M., Blumberg, B., and Inoue, S. (2005) Differential expression of constitutive androstane receptor (CAR), steroid and xenobiotic receptor (SXR) and their target genes, CYP3A4 and CYP2B6 in human prostatic cancer. (submitted to *J. Clinical Cancer Research*).
- Miki, Y., Suzuki, T., Kitada, K., Yabuki, N., Moriya, T., Ishida, T., Ohuchi, N., Blumberg, B., and Sasano, H. (2005) Steroid and xenobiotic receptor (SXR) and its possible regulating gene, organic anion transporting polypeptide-A (OATP-A) in human breast carcinoma. (submitted to *Cancer Research*).

- Tabb, M.M., Verma, S., Zhou, C., and Blumberg, B. (2005) Activation of the steroid and xenobiotic receptor, SXR, induces apoptosis in breast cancer cells. (in preparation for submission to Cancer Research).

CONCLUSIONS

One of the major challenges in breast cancer research is to develop new chemotherapeutic and chemopreventative agents, particularly for non-estrogen dependent breast cancers. SXR activators were able to slow the proliferation of ER⁺ and ER⁻ breast cancer cell lines in culture, and a constitutively active form of SXR was also effective at slowing breast cancer cell growth. We have found that SXR activation is able to induce the expression of the SXR target gene inducible nitric oxide synthase in breast cancer cells. This leads to an increased production of intracellular reactive nitrogen species that has been shown to be a cellular stress that can stabilize and activate the p53 tumor suppressor. In SXR activator-treated breast cancer cells we found that p53 expression as well as expression of the p53 target genes p21 and BAX was increased. SXR activators induced apoptosis in treated breast cancer cells as measured by DNA fragmentation, explaining the decreased cell proliferation in the presence of activated SXR. The activation or stabilization of p53 as a result of cellular stress from increased reactive nitrogen species provides a mechanism to explain the apoptotic response and decreased cell proliferation observed in the presence of SXR activators. Expression of SXR mRNA in ductal carcinomas but not in normal tissue could mean that the presence of SXR is an important prognostic marker for the success of breast cancer treatment with a variety of SXR activating drugs.

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